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Regulation of Laccase Gene Transcription in *Trametes versicolor*

PATRICK J. COLLINS AND ALAN D. W. DOBSON*

Microbiology Department, University College, Cork, Ireland

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The expression of laccase in the white rot fungus *Trametes versicolor* is regulated at the level of gene transcription by copper and nitrogen. We used reverse transcription-PCR to demonstrate that as the concentration of copper or nitrogen in fungal cultures was increased, an increase in laccase activity, corresponding to increased laccase gene transcription levels, was observed. In addition, we demonstrated that the amounts of laccase mRNA and laccase activity in 10-day-old cultures were a direct function of the concentration of either 1-hydroxybenzotriazole, a previously described laccase substrate, or 2,5-xylidine, a well-known laccase inducer, in the medium. No induction was observed after the addition of two aromatic acids, ferulic acid and veratric acid, which have been shown to induce laccase production in other white rot fungi. When either copper, 2,5-xylidine, or both compounds were added to cultures grown in the absence of copper, increased laccase transcript levels were detected within 15 min. Corresponding increases in laccase activity were observed after 24-h incubation only when copper was present.

White rot fungi, such as *Trametes versicolor*, degrade lignin (28, 32, 40) and a broad range of diverse aromatic pollutants (2, 25). This degradation can be accomplished by means of highly reactive radicals (13, 54), the production of which is catalyzed by three groups of oxidative extracellular enzymes, namely, lignin peroxidases (LiP), manganese peroxidases (MnP), and laccases (3, 15, 41). Although both LiP and MnP have been extensively characterized with respect to biochemistry, genetics, and regulation (reviewed in reference 22), much less attention has been focused on laccases. This largely stems from the fact that the model white rot fungus, *Phanerochaete chrysosporium*, has been widely reported to efficiently degrade lignin in the absence of laccase and indeed until recently (50) was thought to be incapable of laccase production. Furthermore, the substrate range of laccases was believed to be limited exclusively to the phenolic subunits of lignin (12, 26, 51) and phenolic pollutants (16, 51), as its oxidation potential would not be high enough to enable it to oxidize nonphenolic structures. However, recent work by Eggert et al. (10) has identified a fungal metabolite of *Pycnoporus cinnabarinus* which mediates degradation of nonphenolic lignin by laccase. In addition, we have observed the oxidation of nonphenolic polycyclic aromatic hydrocarbons, anthracene and benzo[*a*]pyrene, by laccases of *T. versicolor* in the presence of a fungally derived laccase mediator (9). A further indication that laccases may have a more significant role in both lignin and pollutant degradation comes from studies which have identified a number of white rot fungi, including *T. versicolor*, which can effectively degrade lignin without the involvement of LiP (1, 11, 42, 47, 49). Laccases have been implicated as the agents of nonphenolic lignin subunit oxidation by these fungi (10).

Laccases are glycosylated polyphenol oxidases which contain four copper ions per molecule (45). They catalyze the reduction of one dioxygen molecule to two molecules of water, simultaneously oxidizing their aromatic substrates (51). Laccases are produced by the majority of white rot fungi described to date as well as by other types of fungi and by plants. In white rot fungi, they are produced as a number of isozymes (3, 9, 52) encoded by gene families (34, 55). Laccase genes from a number of ligninolytic fungi, including *T. versicolor*, have previously been cloned and characterized (6, 20, 30, 34, 48, 55), and it has been suggested that genes encoding various isozymes are differentially regulated, with some being constitutively expressed and others being inducible (3, 55).

In this study, we investigated the regulation of laccase gene (*lcc*) expression in *T. versicolor* by using reverse transcription (RT)-PCR as a tool to determine the effects on *lcc* mRNA levels when the fungus was cultured under a variety of physiological conditions. We demonstrated that *lcc* transcription was activated by copper and nutrient nitrogen. We also demonstrated that laccase induction by two aromatic compounds, 2,5-xylidine (XYL) and 1-hydroxybenzotriazole (HBT), occurred at the level of gene transcription. The addition of either copper or XYL to 10-day-old cultures grown in the absence of copper resulted in the rapid appearance of *lcc* mRNA, but a corresponding increase in laccase activity occurred only when copper was present.

MATERIALS AND METHODS

Culture conditions. *T. versicolor* 290 (7) was maintained at 4°C on glucose-malt extract slants (5 g of glucose per liter, 3.5 g of malt extract per liter, 15 g of agar per liter). The basal medium used for experimental cultures was previously described (9) without the addition of XYL. Two agar plugs (diameter, 6 mm) from the outer circumference of a fungal colony growing on a glucose-malt extract plate (6 to 8 days) was used as the inoculum. The fungus was grown in 15-ml stationary cultures in 200-ml medical flat bottles (BDH, Poole, United Kingdom) at 26°C in darkness. Bottles were loosely capped to allow passive aeration. In order to determine the point of maximal laccase production, a time course experiment in which laccase activities were measured over a 15-day period was conducted. The effects on *lcc* transcription and laccase activity were determined after the addition of copper (in the form of $CuSO_4$) at various concentrations (0, 0.4, 4, 40, 200, and 400 μM) to this medium. The effects of the addition of another transition metal, zinc (in the form of $ZnSO_4$), at concentrations of 0.4, 4, and 200 μM were also examined. In order to determine the effects of various nutrient nitrogen concentrations on *lcc* expression, ammonium as either ammonium tartrate or ammonium sulfate was provided in the growth medium at concentrations of 0.5, 2.7, 10.9, 27.2, and 54.3 mM. The possibility that molecular oxygen acts as an *lcc* inducer was investigated by purging a set of cultures with 100% oxygen for 5 min on alternate days of incubation. The caps of these bottles were tightly capped. The aromatic compounds XYL, HBT, ferulic acid, and veratric acid (all obtained from Sigma-Aldrich, Dorset, United

* Corresponding author. Mailing address: Microbiology Department, University College, Cork, Ireland. Phone: 353-21-902743. Fax: 353-21-903101. E-mail: a.dobson@ucc.ie.

Kingdom) were tested for the ability to induce *lcc* expression by adding them to the medium at concentrations of 10, 50, 100, and 500 μM before inoculation. Triplicate cultures were analyzed for each datum point after 10 days of incubation. In experiments involving the detection of *lcc* transcripts over time after the addition of copper, XYL, or both, 10-day-old cultures grown in the absence of copper were used. Copper and/or XYL was added to final concentrations of 400 and 500 μM, respectively, and samples were taken at the timepoints indicated.

Laccase activity assays. Laccase activities were determined with 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as the substrate (53). Assay mixtures contained 0.5 mM ABTS, 0.1 M sodium acetate (pH 5.0), and 20- to 100-μl aliquots of culture fluid. Oxidation of ABTS was monitored by determining the increase in A_{420} (ε = 36,000). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per min. Datum points in all cases are averages for triplicate cultures, with standard deviations indicated by error bars.

RNA preparation. Total RNA was prepared by a modification of the method of Gromroff et al. (24). Mycelia from triplicate cultures were separated from culture fluid by being filtered through Miracloth (Calbiochem, Inc., La Jolla, Calif.), washed twice with distilled water, quick frozen in liquid nitrogen, and ground to a powder with a mortar and pestle. Lysis buffer (0.6 M NaCl, 10 mM EDTA, 100 mM Tris-Cl [pH 8.0], 4% sodium dodecyl sulfate, and 50% phenol) was added, and the mixture was shaken vigorously for 20 min and then centrifuged for 10 min at 14,000 × *g*. After a further extraction step with phenol-chloroform-isoamyl alcohol, a 0.75 volume of 8 M LiCl was added, and the mixture was vortexed and incubated overnight at 4°C. RNA was pelleted by centrifugation for 15 min at 14,000 × *g* and resuspended in water. It was precipitated with ethanol, washed with 70% ethanol, and resuspended in water. Residual contaminating DNA was removed by digestion with DNase I (Boehringer GmbH, Mannheim, Germany) according to the manufacturer's protocol. Total RNA was quantitated spectrophotometrically.

RT. Total RNA was used as the template to generate first-strand cDNA in reaction mixtures containing 1 μg of RNA, 40 ng of random hexamer primers (Boehringer), 0.5 mM (each) deoxynucleoside triphosphates, 2 μg of bovine serum albumin, 1× RT buffer (Promega, Madison, Wis.), 200 U of Moloney murine leukemia virus reverse transcriptase (Promega), and 40 U of RNasin ribonuclease inhibitor (Promega). Reaction volumes were adjusted to 20 μl with water. Reaction mixtures were incubated at 37°C for 1 h, and reactions were terminated by heating to 65°C for 10 min.

PCR amplification. Forward (5′-ATTGGCACGGCTTCTTCC-3′) and reverse (5′-GATCTGGATGGAGTCGAC-3′) PCR primers were based on conserved regions between previously described *lcc* sequences from *Trametes* species and closely related fungi (6, 30, 34, 55). The predicted sizes for *lcc* amplification products from genomic DNA and cDNA were approximately 690 and 520 bp, respectively. Sequence analysis of various PCR products indicated the amplification of DNA fragments encoding at least three laccase isozymes, demonstrating the isozyme nonspecificity of the primers. As a result of this nonspecificity, these primers should amplify *lcc* sequences encoding the two major laccase isozymes previously demonstrated to be produced by *T. versicolor* 290 (9). For PCR amplification, a 2-μl volume from each RT reaction mixture was mixed with 75 ng of each primer, 5 μl of 10× KCl-*Taq* buffer (Bioline, London, United Kingdom), 100 μM (each) deoxynucleoside triphosphates, and 1.25 U of *Taq* polymerase. Reaction volumes were adjusted to 50 μl with water. In order to establish that DNA amplifications in all PCRs occurred at similar efficiencies, PCR controls were included in the experiment to examine the effects of various copper concentrations on *lcc* transcription. Each PCR mixture in this experiment was supplied with a fixed amount (50 pg) of a 418-bp genomic LiP (*lip*) fragment from *T. versicolor* 290, cloned into pGEM T (Promega), along with a pair of specific primers to amplify this fragment as previously described (8). This *lip* template should be amplified at a constant level in each PCR. Amplification was performed in a DNA thermal cycler (Omnigene; Hybaid) with a number of cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C), and extension (1 min at 72°C). The number of cycles used was optimized for each experiment to avoid reaching a point at which bands representing different conditions within that experiment would appear to have equal intensities due to having reached a plateau of amplification. The numbers of cycles used in experiments to invest igate the effects of copper, ammonium tartrate, XYL, and HBT on *lcc* mRNA levels were 25, 24, 25, and 20, respectively. In all experiments to examine the effects on transcript levels of the addition of copper, XYL, or both to 10-day-old cultures, 23 cycles of PCR were used.

Aliquots of 10 μl of each reaction mixture were electrophoresed on 2% agarose gels, and the product band intensities within each experiment were visually compared after ethidium bromide staining.

Immunoblot analysis. Extracellular protein samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 4 to 10% polyacrylamide gel (Atto) and transferred to an Immobilon P membrane (Millipore Corp., Bedford, Mass.) electrophoretically. The laccase-specific antibodies used for immunological detection were generated against purified laccase from the basidiomycete PM1 (6). The ProtoBlot Western blot AP system (Promega) was used for detection according to the manufacturer's protocol.

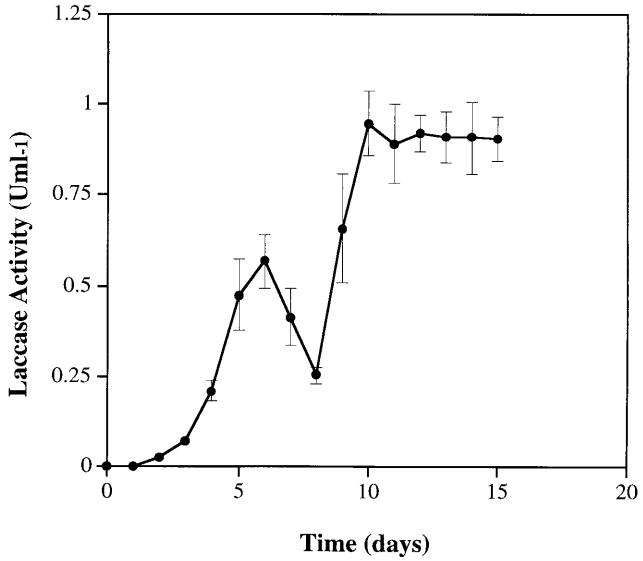

FIG. 1. Time course of laccase activity in the extracellular fluid of a *T. versicolor* culture.

RESULTS

Laccase activity in *T. versicolor* culture. A time course for laccase activity in the extracellular fluid of a *T. versicolor* culture is shown in Fig. 1. Laccase activity was first detected on day 2 of incubation and reached a peak after 6 days. A second and larger activity peak was observed on day 10 of the incubation period, after which the level of laccase activity decreased slightly and remained relatively constant up to day 15. All cultures for subsequent induction studies were therefore harvested after 10 days of incubation.

Regulation of laccase by copper and nitrogen. The induction of *lcc* expression by copper in *T. versicolor* is shown in Fig. 2. Cultures were grown in medium containing copper at various concentrations, and the effects of copper on *lcc* mRNA transcript levels (Fig. 2A) and laccase activities (Fig. 2B) were determined. PCR amplifications of RT reaction products proceeded at uniform efficiencies in all cases, as indicated by the constant level of control *lip* fragment amplification observed in each reaction. In the absence of copper, a low level of *lcc* transcripts was detected in fungal cells; it corresponded to a low level of laccase activity in the culture medium. The addition of 0.4 μM $CuSO_4$ to the growth medium resulted in increased levels of both *lcc* transcripts and enzyme activity. In the presence of increasing $CuSO_4$ concentrations, increases in both *lcc* transcription levels and corresponding enzyme activities were observed, with high levels of both occurring in the presence of 400 μM, the highest copper concentration tested. The laccase activity measured in cultures grown in the presence of 400 μM $CuSO_4$ was approximately 18-fold greater than that in cultures containing no added copper. The correlation observed between copper concentration and *lcc* transcription indicates that copper plays a role in the regulation of *lcc* expression. Zinc, a transition metal which can be substituted for copper in the induction of certain eucaryotic genes (27), was tested for its effects on *lcc* gene transcription. However, the presence of zinc had no detectable effect on *lcc* transcription levels (data not shown).

A direct correlation between the concentration of nutrient nitrogen provided to *T. versicolor* in its growth medium and the level of *lcc* expression was also observed (Fig. 3). Increasing

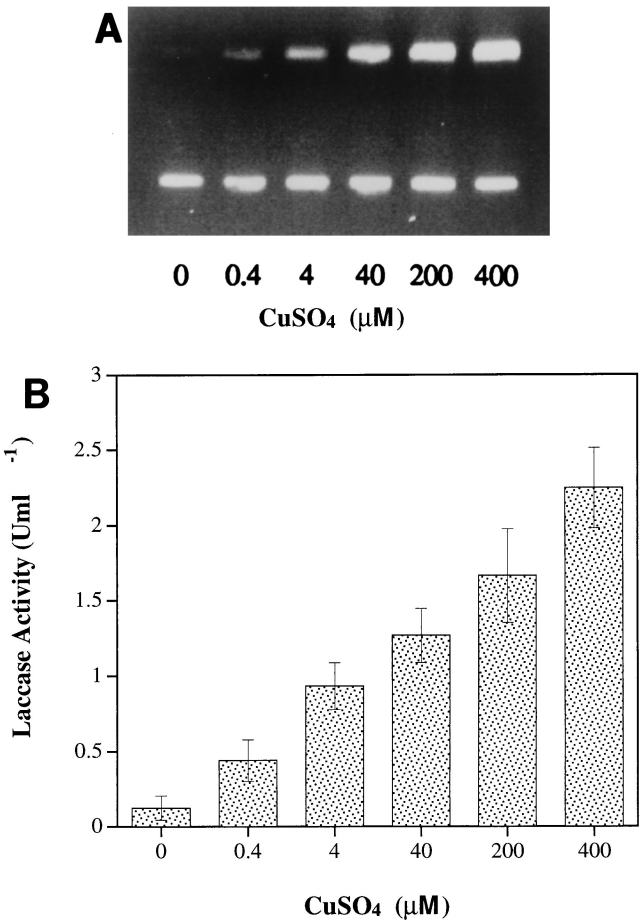

FIG. 2. Induction of *lcc* expression by copper. (A) Effects of various copper concentrations on *lcc* mRNA. The higher-molecular-weight band in each case represents the level of *lcc* transcripts present, and the lower-molecular-weight band represents the *lip* fragment amplified as an internal PCR control. (B) Effects of various copper concentrations on laccase activity.

the concentration of ammonium tartrate resulted in increased levels of *lcc* mRNA (Fig. 3A) and increased laccase activities (Fig. 3B). A similar effect was observed when ammonium sulfate, a noncarbon source, was used as the nitrogen source (data not shown). This indicates that *lcc* expression is regulated at the transcriptional level by nitrogen.

We also investigated the possibility that molecular oxygen has a role in the regulation of *lcc* expression by culturing the fungus in the presence of a high gaseous oxygen concentration. However, no increased *lcc* transcription or laccase activity was observed (data not shown), indicating that oxygen does not function in regulating laccase production.

Induction of laccase by aromatic compounds. Figures 4 and 5 illustrate the effects on *lcc* expression in *T. versicolor* when the fungus was grown in the presence of various concentrations of XYL and HBT, respectively. The observed effects on *lcc* transcription and laccase activity were similar for both compounds. As the concentration of either compound increased, increases in *lcc* mRNA levels (Fig. 4A and 5A) and corresponding enzyme activities (Fig. 4B and 5B) occurred. It is noteworthy, however, that the amount of induction observed at both transcriptional and enzyme activity levels was smaller with HBT than with XYL. The laccase activity in cultures containing 500 μM XYL was 2.3-fold greater than that in corresponding cultures containing HBT. When two other proposed inducers of laccase activity in white rot fungi, ferulic acid (36) and veratric acid (39), were provided in the same range of concentrations as were XYL and HBT, they had no effect on *lcc* mRNA levels or laccase activity (data not shown). The presence of 500 μM or higher concentrations of all four aromatic compounds tested resulted in some inhibition of fungal growth, indicating that these compounds are toxic to the fungus.

Induction by XYL in the absence and presence of copper. Figure 6 illustrates the effects on *lcc* mRNA transcript levels over a 24-h period when copper, XYL, or both were added to *T. versicolor* cultures grown for 10 days in the absence of copper. Very low levels of *lcc* transcription were detectable in cultures before the addition of copper and/or XYL. When copper was added to give a final concentration of 400 μM, increased *lcc* mRNA levels were detected after 15 min (Fig. 6A). Further increases were observed as time progressed, and after 6 h of incubation, high transcript levels were present. The addition of XYL at a concentration of 500 μM, instead of copper, to these cultures resulted in a very similar pattern of transcript appearance (Fig. 6B). In cultures to which copper and XYL were added simultaneously as *lcc* inducers at concentrations of 400 and 500 μM, respectively, an increase in *lcc* transcripts over time was again evident (Fig. 6C). In these cultures, however, copper and XYL appeared to have a cumulative effect on *lcc* transcription, resulting in a greater abundance of *lcc* mRNA being detected after 2 h than when either of the compounds was added individually. These high *lcc* transcript levels were also present after 6 and 24 h of incubation. The laccase activities and extracellular laccase protein levels in

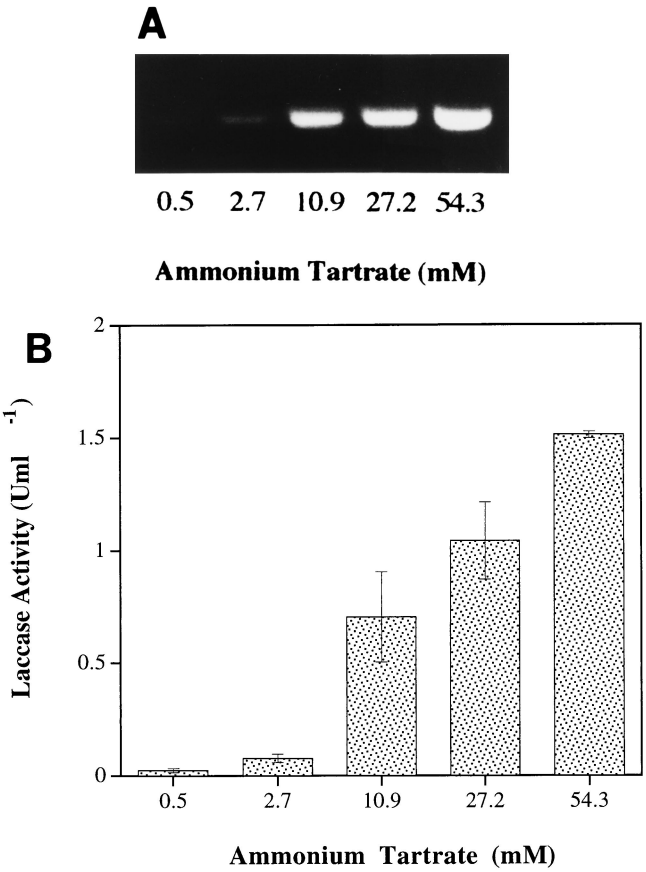

FIG. 3. Induction of *lcc* expression by nitrogen. The effects of various ammonium tartrate concentrations on *lcc* mRNA (A) and laccase activity (B) are shown.

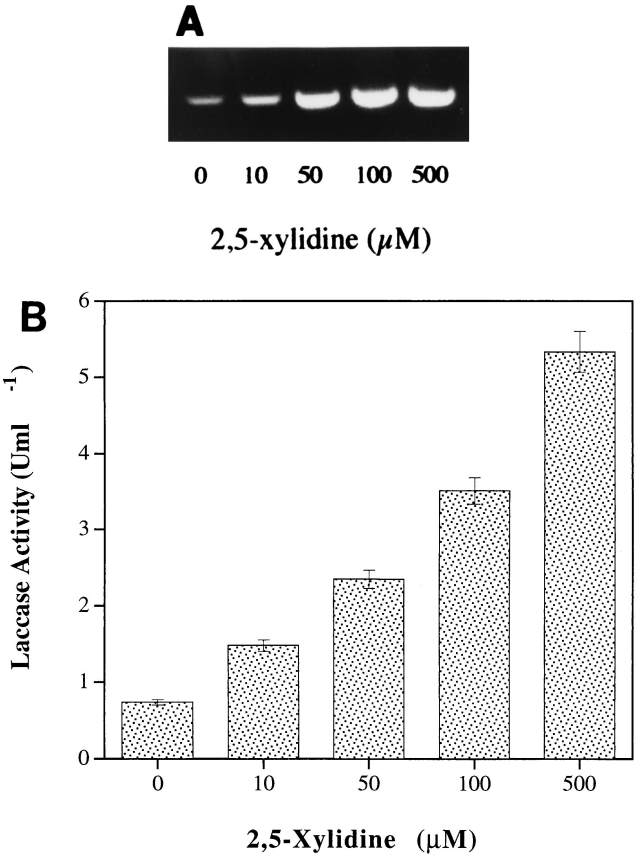

FIG. 4. Induction of *lcc* expression by XYL. The effects of various XYL concentrations on *lcc* mRNA (A) and laccase activity (B) are shown.

cultures induced with XYL in the absence and presence of copper are shown in Fig. 7. The enzyme activities were low in cultures containing copper and in those without copper for the initial 6 h of incubation (Fig. 7A). After 24 h, however, the laccase activities in cultures where copper was present were much higher than those in cultures which contained no copper, even though high *lcc* mRNA levels were present at this time-point for both sets of cultures (Fig. 6). Immunoblot analysis (Fig. 7B) indicated that similar levels of laccase protein were present after 24 h in cultures containing XYL alone as the inducer and in those containing both XYL and copper. This observation demonstrates that although XYL can have a marked effect on the induction of *lcc* gene transcription and these transcripts are efficiently translated, no increased laccase activity can be detected unless copper is present.

DISCUSSION

Although laccase production in various white rot fungi is known to be influenced by a number of physiological factors (3, 11), little work has been done to examine the regulation of *lcc* expression at the molecular level. In contrast, the regulatory mechanisms involved in the expression of LiP and MnP, the other ligninolytic oxidative enzymes, have been studied in detail (22, 37, 44). In this work, we used RT-PCR to investigate the induction of *lcc* expression in *T. versicolor* when the fungus was cultured under a variety of physiological conditions and in the presence of known *lcc* inducers.

Copper regulates *lcc* transcription in *T. versicolor*. As the copper concentration in the growth medium increased, increased levels of *lcc* mRNA transcripts were seen, with concomitant increases in laccase activities. Considering that *T. versicolor* laccase contains four copper ions which are essential for activity (45), this finding is not surprising. Froehner and Eriksson (17) also observed decreased laccase production by the ascomycete *Neurospora crassa* when copper was removed from its growth medium. This regulation of *lcc* expression by copper is analogous to the effect of manganese on MnP gene (*mnp*) expression (4). Although the precise mechanism by which manganese induces *mnp* transcription is unknown, a number of putative metal response elements (MREs) have been identified in promoter regions of *mnp* genes (22). These putative MREs conform exactly to the consensus sequence, TGCRCNC, found in the promoters of metallothionein genes in higher eucaryotes (27). The expression of metallothionein genes in eucaryotic organisms is induced by a range of heavy metals (27). In both higher and lower eucaryotes, this regulation operates via a single-component system, whereby a single intracellular metalloregulatory protein functions as both the metal receptor and the *trans*-acting transcription factor (27). Coll et al. (6) have identified a region in the *lcc* promoter from the basidiomycete PM1 which has some similarity with the binding site for the ACE1 transcription factor in the *Saccharomyces cerevisiae SOD1* gene (23). This gene encodes a Cu-Zn superoxide dismutase which is regulated by copper and zinc. We have analyzed the promoter sequences upstream of the TATA boxes in *lcc* genes from a number of other white rot fungi (20, 30, 34, 48, 55) and can identify no sequences similar to the ACE1 binding site of *S. cerevisiae SOD1* or to the MREs

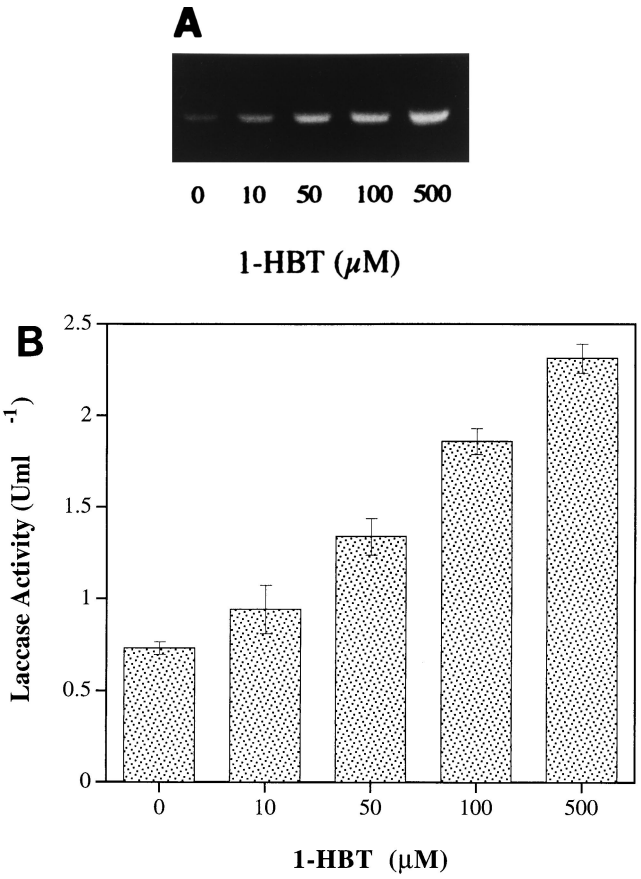

FIG. 5. Induction of *lcc* expression by HBT. The effects of various HBT concentrations on *lcc* mRNA (A) and laccase activity (B) are shown.

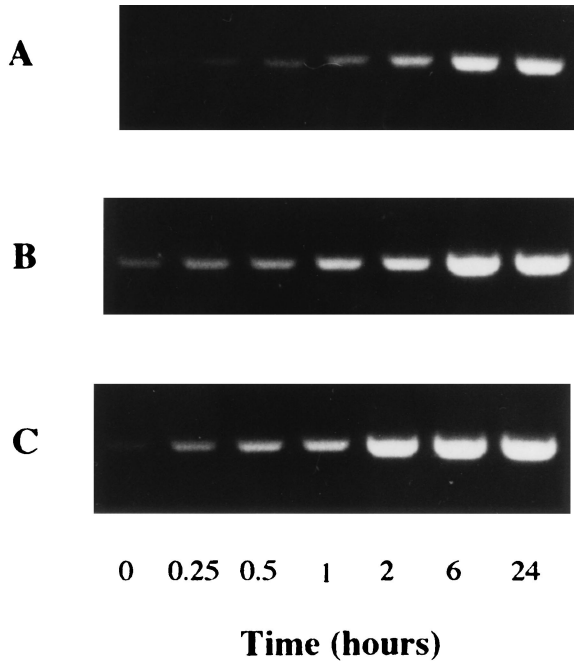

FIG. 6. Time course of induction of *lcc* expression by copper, XYL, or both. The appearance of *lcc* mRNA over time after the addition of 400 μM $CuSO_4$ (A), 500 μM XYL (B), or 400 μM $CuSO_4$ and 500 μM XYL (C) is shown.

of higher eucaryotes. This leads us to speculate that the mechanism involved in the copper regulation of *lcc* transcription is unrelated to the mechanisms which mediate the regulation of metallothionein gene expression. Further evidence for a distinct mechanism of *lcc* regulation by copper comes from our finding that the presence of zinc in fungal cultures failed to induce *lcc* transcription.

LiP and MnP production in *P. chrysosporium* is well-known for being stimulated by limiting nitrogen concentrations, and this has been shown to involve regulation at the level of gene transcription (38, 43). In contrast, higher LiP and MnP titers have been observed in other white rot fungal species in the presence of high concentrations of nitrogen (7, 31). Eggert et al. (11) have shown that laccase activities in culture fluids of *Pycnoporus cinnabarinus* are also dependent on the nitrogen concentration. In this study, we demonstrated that nitrogen induced laccase production in *T. versicolor* at the level of gene transcription. When nutrient nitrogen was provided to the fungus at increasing concentrations, corresponding increases in *lcc* mRNA and laccase activity were observed. Hence, it seems that nitrogen is an important factor in regulating expression of the three major ligninolytic enzymes in white rot fungi.

We investigated whether oxygen may also have a role in the regulation of *lcc* transcription. Laccase uses molecular oxygen as its primary substrate, reducing it to two molecules of water, in the process of oxidizing its aromatic substrate (51). Oxygen has also been found to have an enhancing effect on lignin degradation by white rot fungi (33, 35). Furthermore, Li et al. have demonstrated that in the presence of manganese, molecular oxygen induces *mnp* expression (37). Our results indicate, however, that purging *T. versicolor* cultures with 100% oxygen on alternate days of growth has no effect on either *lcc* mRNA or laccase activity levels.

It has long been established that the addition of XYL to cultures of *T. versicolor* or other basidiomycetes has a large stimulatory effect on laccase production (3, 15). Although some laccase isozymes in *Trametes* species are inducible by XYL, others are not (3), and it has previously been demonstrated that in *Trametes villosa*, this induction is at the level of gene transcription (55). Low-molecular-weight aromatic acids, such as ferulic acid (36), veratric acid (39), and gallic acid (21), which are structurally related to lignin have also been shown to induce laccase production in a range of other fungi. In this study, we investigated the abilities of three previously reported laccase inducers, XYL, ferulic acid, and veratric acid, as well as HBT, previously reported to be a mediator in laccase catalysis (5, 29), to induce *lcc* transcription in *T. versicolor*. Our findings indicate that XYL (Fig. 4) and HBT (Fig. 5) can effectively activate *lcc* transcription; no induction was observed in the presence of either ferulic acid or veratric acid. The inability of aromatic acids to induce *lcc* expression in *T. versicolor* 290 is perhaps not surprising since previous work has indicated that veratric acid also fails to induce increased laccase activity levels in another strain of *T. versicolor* (39).

The mechanisms by which the aromatic compounds used in this study activate *lcc* transcription in various fungi have yet to be determined. These compounds are toxic to fungi, and in this study, we observed that when any of these compounds was present in *T. versicolor* culture fluids at a concentration in excess of 2 mM, no fungal growth occurred. Thurston (51) has suggested that one function of laccases is to detoxify highly reactive aromatic compounds by polymerizing them. Therefore, it is possible that *lcc* induction by XYL and HBT is indicative of a response developed by fungi to oxidize and hence reduce the potentially toxic effects of such compounds.

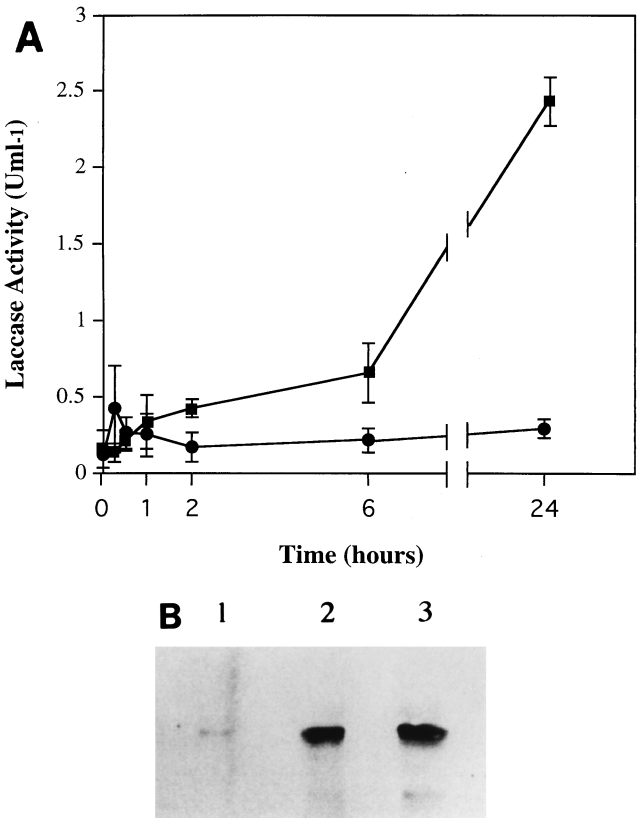

FIG. 7. (A) Time course of laccase activity after the addition of 500 μM XYL (●) or 400 μM $CuSO_4$ and 500 μM XYL (■). (B) Immunoblot analysis of extracellular proteins (5 μg per lane). Lanes: 1, no addition of XYL or copper; 2, 24 h after the addition of XYL; 3, 24 h after the addition of XYL and copper.

Indeed, in the case of XYL-induced cultures of *T. versicolor*, laccase production coincided with the formation of a dark precipitate (data not shown) which may represent a laccase-polymerized form of the inducer.

The induction of laccase production in various fungi appears to be specific for certain aromatic compounds; consequently, some specific mechanisms of transcriptional activation are likely to be involved. An investigation of the mechanism of induction of mammalian cytochrome P-450c in response to aromatic hydrocarbons revealed xenobiotic responsive elements (XREs) in the cytochrome P-450c promoter region (18). The XRE receptor or binding protein is a member of a large family of regulatory proteins which activate gene transcription in response to the presence of nonpolar carbon compounds (14, 18, 19). An XRE has been identified in the promoter of a *lip* gene (46); it conforms to the XRE consensus sequence, TCACGC (19). We have analyzed a number of white rot fungal *lcc* promoters (6, 20, 30, 34, 48, 55) and have found putative XREs in two of them. The *lcc* promoter in the basidiomycete PM1 (6), a closely related fungus to *T. versicolor*, contains a sequence identical to the XRE consensus 180 bp upstream of the TATA box. The sequence, TCACGG, which closely matches the consensus is present 191 bp 5′ to the TATA box in the *lcc* promoter in *Pleurotus ostreatus* (20). The presence of these putative XREs suggests that transcription of these *lcc* genes is indeed activated by aromatic compounds, such as those utilized in this study. A reason for the absence of putative XREs from promoters of other white rot fungal *lcc* genes may be that these genes encode laccase isozymes which are noninducible by such aromatic compounds.

The addition of either copper, XYL, or both to copper-deficient cultures of *T. versicolor* resulted in the immediate activation of *lcc* transcription and the rapid accumulation of *lcc* mRNA. The fact that XYL can induce *lcc* transcription in the absence of copper indicates that copper is not an absolute requirement for activation of *lcc* transcription. However, when copper and XYL were simultaneously present as *lcc* inducers, they acted synergistically to activate *lcc* transcription at a rate faster than that for either separately. The extracellular laccase protein levels appeared to be similar after 24 h in cultures induced with XYL alone and in those induced with XYL and copper. Although this production of laccase protein correlated with an increase in laccase activity in cultures where copper was present, no increased activity was detected in cultures which did not contain copper. The likely explanation is that the enzyme remains inactive in these cultures because copper is not available for incorporation to produce functional laccase protein.

In conclusion, *lcc* expression in *T. versicolor* appears to be regulated by a range of factors at the level of gene transcription. Further work to investigate the differential expression of genes encoding individual laccase isozymes in *T. versicolor* is planned. In addition, efforts are being made to determine the precise mechanisms of *lcc* transcriptional activation by nutrient nitrogen, copper, and a number of aromatic inducers and the associations among these mechanisms in *T. versicolor* and a range of other white rot fungi.

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